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Differential Sensitivity of Agonist- and Antagonist-occupied Gonadotropin-releasing Hormone Receptors to Protein Kinase C Activators

A MARKER FOR RECEPTOR ACTIVATION*

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Gonadotropin-releasing hormone (GnRH) stimulates release of pituitary gonadotropins by activating specific plasma membrane receptors. In the present studies, we have used activators of the Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) to probe the binding characteristics of agonist- or antagonist-occupied GnRH receptors in intact cell cultures, using a radioligand receptor assay. Specific binding of [^{125}I-Tyr^5,D-Ser(tBu)^6,Pro^9,NHEt]GnRH (Buserelin), a high-affinity GnRH agonist, was increased to 180% of control in the presence of 150 nM phorbol 12-myristate 13-acetate (PMA) or 100 nM phorbol 12,13-dibutyrate (PDB), and to 125% of control in the presence of 200 μM 1,2-dioctanoylglycerol, after 20 min at 23 °C. The PMA effects were associated with apparent increases in both binding affinity and number of binding sites. The effects of protein kinase C activators on Buserelin binding were concentration- and time-dependent and were not seen with 4α-PMA or 1,2-dioctanoyl-3-Cl-glycerol, neither of which activate protein kinase C. In contrast, PMA had no measurable effects on specific binding of a GnRH receptor antagonist, Ac[D-pCl-Phe^{1,2},D-Trp^3,^{125}I-Tyr^5,D-Lys^6,D-Ala^{10}]GnRH. When cell cultures were pretreated with 100 nM PDB in the absence of GnRH and then washed to remove the phorbol ester, no effects of prior protein kinase C activation were detected upon subsequent addition of Buserelin. However, when PDB pretreatment was carried out in the presence of 0.3 μM GnRH, residual enhancement of Buserelin binding, but not antagonist binding, was observed at either 23 or 4 °C. The radiolabeled agonist activated, and the antagonist blocked, GnRH receptor-mediated luteinizing hormone release and [^{3}H]inositol phosphate production in cells preloaded with [^{3}H]inositol. These findings suggest that the action of protein kinase C on the GnRH receptor, either direct or indirect, requires the receptor to be in an activated (agonist-occupied) state but does not require receptor internalization. The mechanism of these effects on GnRH agonist binding is not known but may involve sequestration of surface receptors, expression of new receptors, and/or modulation of GnRH receptor affinity.

Gonadotropin-releasing hormone (GnRH)[1] is a hypothalamic decapeptide which stimulates Ca^{2+}-dependent release of the pituitary gonadotropins, LH and follicle-stimulating hormone, by activating specific plasma membrane receptors (1). The availability of numerous agonist and antagonist analogs of GnRH has aided both our understanding of the mechanism of GnRH action and development of the ability to regulate the GnRH receptor and cellular responsiveness (2, 3). Chronic down-regulation of pituitary GnRH receptors, for example, is considered to account, in part, for the reduction in circulating levels of gonadotropins which accompanies prolonged clinical treatment with metabolically stable GnRH agonist analogs (4). Following occupancy by an agonist, GnRH receptors undergo patching, capping, and internalization (5–7) by a cytoskeleton-dependent mechanism (8). Antagonist analogs of GnRH, which appear to occupy the same receptor sites as agonist analogs (9), are also internalized but at a slower rate than are agonists (10, 11). Down-regulation of GnRH antagonist-occupied receptors can be produced, however, when these receptors are artificially cross-linked by a divalent antibody (12), a procedure which also activates other GnRH receptor-mediated events (13, 14).

GnRH, like other Ca^{2+}-linked extracellular messengers, provokes production of diacylglycerols (15) and redistribution of the Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C, 16–18) in target cells. However, the presence of this enzyme activity does not appear to be required for GnRH-stimulated LH release (19) or for homologous receptor down-regulation and desensitization (20). In other experimental systems (21, 22), protein kinase C activators have been shown to alter receptor-binding characteristics, and we have demonstrated the ability of these compounds to uncouple agonist-activated GnRH receptors from inositol phospholipid hydrolysis (14). In the present studies, we have used exogenous activators of protein kinase C to further examine the properties of the agonist-occupied GnRH receptor. Using a radioligand-binding assay which permits measurement of GnRH receptors in intact cells in culture (20), we show that GnRH agonist and antagonist binding are differentially sensitive to modulation by activators of protein kinase C.

EXPERIMENTAL PROCEDURES

Materials—Medium 199, horse and fetal bovine sera (Irvine Scientific, Santa Ana, CA), collagenase (Serva, Heidelberg, Federal

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[1] The abbreviations used are: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; BSA, bovine serum albumin (Cohn fraction V); PMA, phorbol 12-myristate 13-acetate (4β-isomer unless otherwise specified); PDB, phorbol 12,13-dibutyrate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

Republic of Germany), hyaluronidase Type II, DNAse I Type II, bacitracin, EGTA, gentamycin sulfate, bis-benzimide, calf thymus DNA, Dowex-1 X8 (Sigma), BSA, HEPES (Boehringer Mannheim), and Sephadex G-25 (Pharmacia LKB Biotechnology Inc.) were obtained as indicated. Na[^{125}I] (1760 Ci/mmol), [20-^{3}H]phorbol 12,13-dibutyrate (19.7 Ci/mmol), and *myo*[2-^{3}H]inositol (17.9 Ci/mmol) were obtained from Amersham Corp. Native sequence GnRH was obtained from the National Pituitary Agency. The GnRH superagonist analog [D-Ser(tBu)^{6},Pro^{9},NHEt]GnRH (Buserelin, 23) was obtained from Hoescht-Roussel Pharmaceuticals (Sommerville, NJ), and the pure antagonist analog Ac[D-pCl-Phe^{1,2},D-Trp^{3},D-Lys^{6},D-Ala^{10}]GnRH (DC-12-119) was generously provided by Dr. David Coy, Tulane University, New Orleans, LA (24). The GnRH analogs were iodinated using chloramine T (25, 26), and the radioligands were purified by chromatography on Sephadex G-25 in 50 mM acetic acid, 0.1% (w/v) BSA (27). Specific radioactivity of the labeled analogs ranged from 1000–1400 Ci/mmol, as determined by self-displacement (27) in specific radioimmunoassays, using antisera prepared and characterized in our laboratory. Phorbol esters (LC Services, Woburn, MA) were prepared as 100-fold concentrated stock solutions in dimethyl sulfoxide. Diacylglycerols were synthesized as described (28), stored dry under N_2 at −70 °C, and prepared as 20 mM stock solutions in ethanol immediately prior to use. Final vehicle concentration in all treatment solutions was 1% (v/v) and had no detectable effect on binding activity, LH release, or [^{3}H]inositol phosphate production.

Preparation of Pituitary Cell Cultures—Pituitary glands were obtained from female weanling Sprague-Dawley rats (Sasco, Omaha, NE). Dispersed cells were prepared as described previously (29) by incubation of minced pituitaries at 37 °C in 10 ml medium 199 containing 0.3% BSA and 10 mM HEPES, pH 7.4 (M199/BSA), with 1.25 mg/ml collagenase and 1.0 mg/ml hyaluronidase. DNase I (10 μg/ml) was added during the final 15 min of incubation. Dispersed cells were filtered through organza cloth, collected by centrifugation at 150 × *g*, and resuspended (cells from 1–1.5 pituitary/2 ml) in M199/BSA containing 10% (v/v) horse serum and 2.5% fetal bovine serum. Two-milliliter aliquots of the cell suspension were placed in 22-mm diameter wells of 12-well plastic culture plates (Costar, Cambridge, MA). Cells were maintained in culture at 37 °C in a water-saturated atmosphere for 2–3 days. All media for cell preparation and culture were sterilized by filtration through 0.45-μm membranes (Millipore, Bedford, MA) immediately prior to use.

GnRH Receptor Binding—GnRH receptor-binding assays in intact cells were conducted at 23 °C by a modification of the method previously described (20). Briefly, culture wells were washed twice with 2 ml of M199/BSA at 23 °C to remove serum components and unattached cells. Radioligands were then added in a final volume of 0.5 ml of M199/BSA containing 1 mM bacitracin and protein kinase C activator or vehicle. After 20 min (except where indicated), the binding incubations were terminated by decanting the radioligand solution and rapidly washing the wells with 1 ml of M199/BSA at 4 °C. Cells were then scraped from the plates in 2 ml of M199/BSA containing 2.5 mM EGTA at 4 °C and were collected by centrifugation (3000 × *g*, 4 °C for 10 min) through a cushion of 1 ml of M199/BSA, 0.3 M sucrose. Radioactivity in the cell pellets was determined by gamma spectroscopy (Beckman 5500 gamma counter). Specific binding was determined by subtracting nonspecific binding (assays containing 10 μM unlabeled GnRH) from total binding. Dissociation constants (K_d) and maximum numbers of binding sites (B_{max}) were estimated from Scatchard analysis of data from saturation experiments (30). Regression lines were determined by the method of least squares. DNA was determined in homogenized cell pellets by the bis-benzimide fluorescence method (31), using calf thymus DNA as a standard.

Measurement of LH Release and Total [^{3}H]Inositol Phosphate Production—Pituitary cells were enzymatically dispersed, resuspended in serum-containing medium (cells from 1 pituitary/4 ml), and plated (1 ml of cell suspension/16-mm well of 24-well culture plates). After 2–3 days in culture, cells were preloaded with [^{3}H]inositol for 5 h at 37 °C as described previously (14, 19). Cells were then washed twice with 1 ml/well M199/BSA containing 5 mM LiCl (M199/BSA/LiCl) and were challenged (90 min at 37 °C) with the indicated compounds in 0.5 ml/well M199/BSA/LiCl. These conditions provide maximal production and stabilization (32) of the GnRH-stimulated inositol phosphate signal in pulse-labeled pituitary cell cultures (33). Incubations were terminated by removing the challenge media and covering the cells with 1 ml/well 0.1 M formic acid at 4 °C. Cells were lysed by one freeze-thaw cycle (−40 °C), and total [^{3}H]inositol phosphates in cell lysates were determined by Dowex anion exchange chromatography and liquid scintillation spectroscopy as described (14, 19). The LH released into challenge media was determined by radioimmunoassay, using highly purified rat LH (LH I-6) for iodination (25) and reference preparation (RP2) obtained from the National Institute of Diabetes and Digestive and Kidney Diseases, and specific antiserum (C-102) prepared and characterized in our laboratory (34). Bound and free hormone were separated using immobilized protein A (35). Where necessary, [^{3}H]inositol phosphate and radioimmunoassay data were corrected for the presence of iodinated Buserelin or GnRH antagonist.

Phorbol Ester Binding and Washout—Phorbol ester binding in intact cell cultures was measured by a procedure analogous to that employed for GnRH receptor binding (36). Pituitary cells were dispersed, maintained in culture for 2–3 days, and washed as described above. The binding incubation was initiated by addition of 0.5 ml of M199/BSA containing 100 nM (~1 μCi) [^{3}H]PDB. After 20 min at 23 °C, cells were washed, scraped from plates, and collected by centrifugation as described above for GnRH receptor assay. Cell pellets containing bound radioligand were resuspended in 0.4 ml of water and transferred to vials for determination of radioactivity by liquid scintillation spectroscopy. Specific binding was determined by subtracting nonspecific binding (incubations containing 1 μM unlabeled PMA) from total binding. In order to estimate the efficiency of PDB washout, cells which had been incubated for 20 min with 100 nM [^{3}H]PDB were immediately washed twice with 2 ml/well M199/BSA at 23 °C. The cells were then washed with 1 ml/well M199/BSA at 5-min intervals for a total of 30 min. Following this washout period, cells were washed, scraped from plates, and collected for radioactivity determination as described above. DNA was determined in cell pellets derived from identically treated cell cultures. Under these conditions, the initial specific [^{3}H]PDB binding (20 fmol/μg DNA) was reduced by >90% after washout; the washout procedure did not significantly reduce the amount of DNA recovered in cell pellets.

Other Methods—Differences between treatment and control groups were evaluated using Student's *t* test; $p < 0.05$ was considered significant.

RESULTS

As we recently reported (20), ^{125}I-Buserelin exhibited saturable and high-affinity specific binding to intact pituitary cells attached to culture plates, with Scatchard analysis indicating the presence of a single class of GnRH-binding sites (Fig. 1). In the presence of 15 nM PMA, a phorbol ester which activates protein kinase C, specific binding of Buserelin was

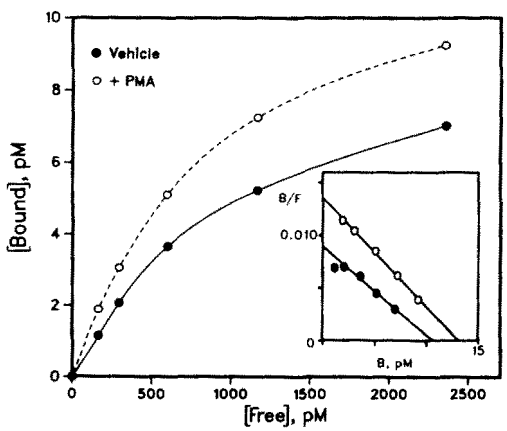

FIG. 1. **Saturable specific binding of Buserelin to pituitary cells in culture.** Pituitary cell cultures were prepared and Buserelin specific binding measured by coincubation of radioligand with either vehicle (●) or 15 nM PMA (○) as described under "Experimental Procedures." Scatchard analysis (*inset*) of the data shown revealed a K_d of 1.2 nM and B_{max} of 10.7 pM (1.2 fmol/μg DNA). Data shown (means of duplicate culture wells) are from a representative experiment; data pooled from four separate experiments revealed a K_d of 980 ± 140 pM (mean ± S.E.) for Buserelin. Nonspecific binding ranged from 3–10% of total binding in control cultures and was not affected by PMA. B_{max} values determined with Buserelin did not differ significantly from those reported previously (20).

significantly enhanced (Fig. 1). Scatchard analysis of data from four separate experiments showed that the enhancement of Buserelin binding was associated with a minor but significant increase in apparent number of binding sites (116 ± 3% of control, mean ± S.E.) and decrease in apparent K_d (80 ± 3% of control). Effects of similar magnitude were observed when PMA was added 5 or 30 min prior to addition of Buserelin or when the binding incubation was conducted at 37 °C (not shown).

PMA and PDB (another protein kinase C-activating phorbol ester) increased Buserelin binding in a concentration-dependent fashion (Fig. 2), with half-maximal effects occurring at 5–10 nM phorbol ester. Data pooled from 18 separate experiments revealed that specific binding of Buserelin (added at approximately K_d concentration) was increased to 146 ± 5% (mean ± S.E.) of vehicle control in the presence of 15 nM PMA. In addition the effect of PMA on Buserelin binding was specific, since the 4α-isomer of PMA, which does not activate protein kinase C *in vitro*, had no measurable effect on Buserelin binding to pituitary cells (Fig. 2). Specific binding of Buserelin also was enhanced in a concentration-dependent fashion by a protein kinase C-activating synthetic diacylglycerol, 1,2-dioctanoylglycerol (Fig. 3). Specific binding of Buserelin (added at approximately K_d concentration) was increased to 125 ± 2% (mean ± S.E., $n = 3$ separate experiments) of vehicle control in the presence of 200 μM dioctanoylglycerol. 1,2-Dioctanoyl-3-Cl-glycerol, which does not activate protein kinase C, also had no measurable effect on Buserelin binding.

The time course of Buserelin binding in the presence of PMA is shown in Fig. 4. As previously observed (20), Buserelin binding in control cultures was maximal after 20–30 min of incubation. Likewise, the enhanced level of binding in the presence of PMA was maximal after 20–30 min of incubation. In other experiments, the reversibility of Buserelin binding was assessed by replacing Buserelin-containing medium, after a 20-min binding incubation at 23 °C, with medium containing 10 μM unlabeled GnRH for 3 h at 4 °C, in order to displace accessible bound ligand. These experiments showed that 30–40% of the ligand bound in the absence of PMA was not displaced by excess GnRH, while 70% of radioligand initially bound in the presence of 15 nM PMA was resistant to subsequent displacement.

In order to confirm that the Buserelin radioligand used to measure GnRH receptors was able to activate receptor-dependent responses, the ability of iodinated Buserelin to provoke LH release and inositol phosphate production in cell cultures was investigated. As shown in Fig. 5, iodinated Buserelin stimulated concentration-dependent increases in both LH release and [^{3}H]inositol phosphate production, with maximal efficacies equivalent to GnRH. In addition, Buserelin-stimulated inositol phosphate production was inhibited in the presence of 15 nM PMA, indicating the ability of PMA to uncouple receptor activation by Buserelin from inositol phospholipid hydrolysis.

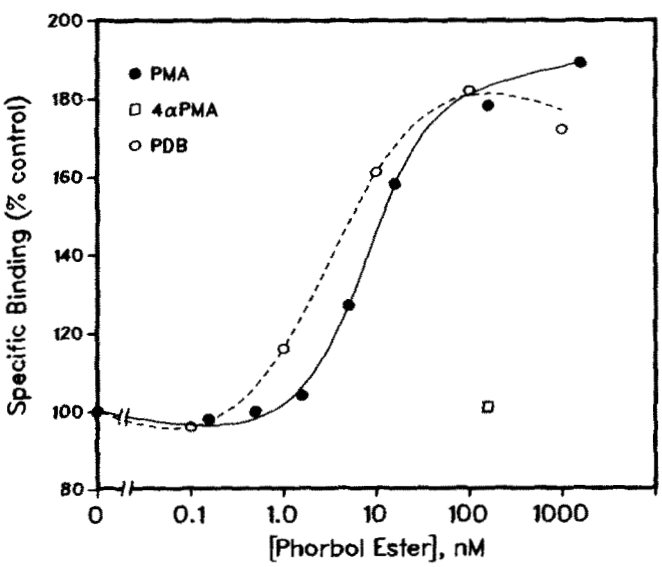

FIG. 2. **Concentration dependence and specificity of phorbol ester enhancement of Buserelin binding.** Specific binding of Buserelin, added at approximately K_d concentration (0.8–1.0 nM), was measured by coincubation of radioligand with the indicated concentrations of either PMA (●), 4α-PMA (□), or PDB (○). Results are expressed as a percent of specific binding measured in the absence of phorbol ester. Phorbol esters had no effect on nonspecific binding. Data shown are the mean of four separate experiments, each consisting of 2–4 replicate treatment wells, which agreed within 7% of mean values.

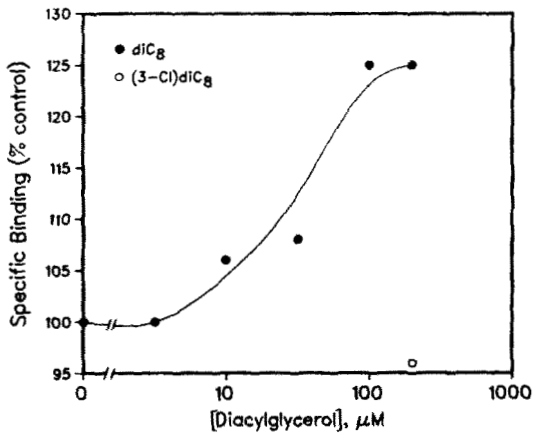

FIG. 3. **Concentration dependence and specificity of diacylglycerol enhancement of Buserelin binding.** Specific binding of Buserelin, added at approximately K_d concentration (0.8–1.0 nM), was measured by coincubation of radioligand with the indicated concentrations of dioctanoylglycerol (diC_8) (●) or 1,2-dioctanoyl-3-Cl-glycerol ($3\text{-}Cl)diC_8$ (○). Results are expressed as a percent of specific binding measured in the absence of diacylglycerol. The diacylglycerols had no effect on nonspecific binding. Data shown are means of three separate experiments, each consisting of 2–4 replicate treatment wells, which agreed within 6% of mean values.

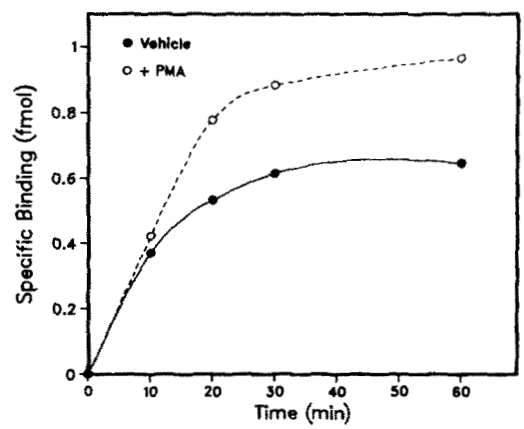

FIG. 4. **Time course of Buserelin binding in the presence of PMA.** Specific binding of Buserelin, added at 1.0 nM, was measured at the indicated times after addition of radioligand at 23 °C in the presence of vehicle (●) or 15 nM PMA (○). Data shown (mean of four replicate treatment wells, agreeing within 5%) are from one of two separate experiments.

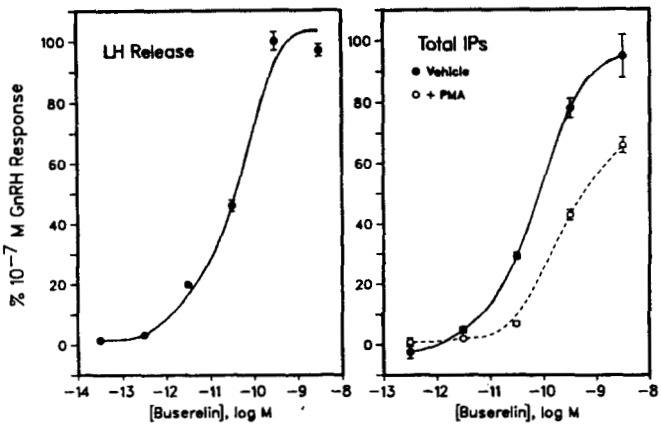

FIG. 5. **Stimulation of LH release and [^{3}H]inositol phosphate (*IP*) production by GnRH agonist radioligand.** Pituitary cell cultures were prepared and preloaded with *myo*[2-^{3}H]inositol as described under "Experimental Procedures." Cells were then challenged with the indicated concentrations of iodinated Buserelin or with 100 nM GnRH in M199/BSA/LiCl in the presence of vehicle (●) or 15 nM PMA (○) for 90 min at 37 °C. Results (mean ± S.E., n = 3 treatment wells from one of two replicate experiments) are expressed as a percent of the response to 100 nM GnRH (maximal efficacy).

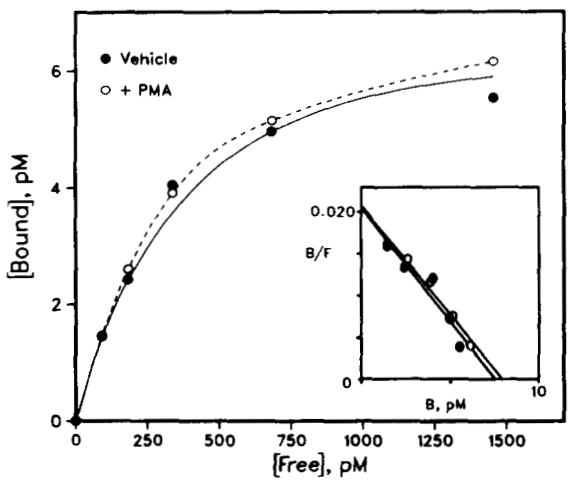

FIG. 6. **Saturable specific binding of GnRH antagonist to pituitary cells in culture.** Pituitary cell cultures were prepared and radioligand antagonist-specific binding measured by coincubation of radioligand with vehicle (●) or 15 nM PMA (○) as described under "Experimental Procedures." Scatchard analysis (*inset*) of the data shown revealed a K_d of 0.37 nM and B_{max} of 7.6 pM (0.9 fmol/μg DNA) in the absence of PMA. Nonspecific binding ranged from 15–45% of total binding and was not affected by PMA. Data shown (means of duplicate culture wells) are from one of three separate experiments. B_{max} values determined with the GnRH antagonist did not differ significantly from those reported previously for Buserelin binding (20).

When a radiolabeled GnRH antagonist was used to measure GnRH receptors in intact cells, specific binding again was found to be saturable and of high affinity (Fig. 6). In contrast to the GnRH agonist binding, however, PMA had no measurable effect on antagonist binding. Using data pooled from three separate experiments, specific binding of the antagonist (added at approximately K_d concentration) in the presence of 15 nM PMA was 94 ± 6% (mean ± S.E.) of vehicle control. Addition of PMA 30 min prior to addition of the antagonist radioligand had no effect on binding (not shown). The ability of the iodinated antagonist to occupy GnRH receptors in a nonactivating manner was confirmed by the concentration-dependent inhibition of GnRH-stimulated LH release and

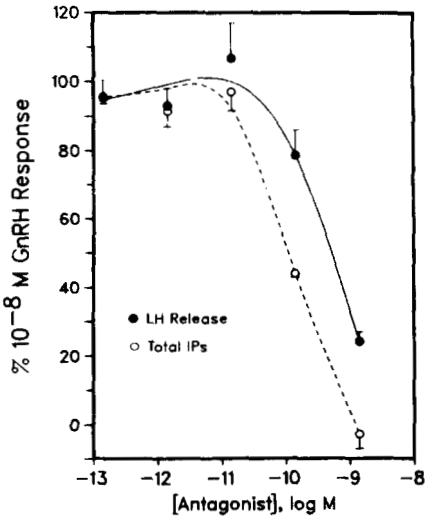

FIG. 7. **Blockade of GnRH-stimulated LH release and [^{3}H] inositol phosphate production by GnRH antagonist radioligand.** Pituitary cell cultures were prepared and preloaded with *myo*[2-^{3}H]inositol as described under "Experimental Procedures." Cells were then challenged with 10 nM GnRH in the presence of the indicated concentrations of iodinated GnRH antagonist in M199/BSA/LiCl. Results (mean ± S.E., n = 3 treatment wells from one of two replicate experiments) are expressed as a percent of the response to GnRH in the absence of antagonist. The antagonist alone (1.5 nM) had no measurable effect on either LH release or [^{3}H]inositol phosphate levels.

[^{3}H]inositol phosphate production by the antagonist radioligand (Fig. 7).

In order to test the requirement for the simultaneous presence of GnRH agonist and protein kinase C activator in the development of enhanced agonist binding, cells were pretreated with a maximally effective concentration of PDB (100 nM) or with vehicle, then were washed to remove the phorbol ester, and finally were incubated with GnRH receptor radioligand in the presence or absence of PDB. As shown in Fig. 8*A*, cells pretreated with vehicle exhibited the characteristic increase in Buserelin binding in the presence of PDB. In cells pretreated with 100 nM PDB, Buserelin binding was not significantly different ($p > 0.2$) compared to vehicle-pretreated controls. Similarly, PDB pretreatment had no effect on the binding of the antagonist radioligand. The PDB-pretreated cells remained fully responsive to PDB when the phorbol ester was added back with the radioligand agonist. In contrast, cells pretreated with 100 nM PDB plus 0.3 μM GnRH (Fig. 8*B*) exhibited a significant increase in Buserelin binding (117 ± 2% of control) compared to cells pretreated with GnRH alone. However, no change in binding of the antagonist radioligand was found after PDB plus GnRH pretreatment.

The residual effects on Buserelin (agonist) binding, but not antagonist binding, following PDB plus GnRH pretreatment suggest that, unlike the actions of phorbol esters reported in other systems (21, 22), phorbol esters may not provoke a net internalization of GnRH receptors. As a further test of the possible role of receptor internalization in the actions of PDB on Buserelin binding, cells which had been pretreated and then washed as described for Fig. 8 were evaluated for agonist and antagonist binding at 4 °C (Fig. 9). Binding of Buserelin at 4 °C, as at 23 °C, was increased significantly (130 ± 1% of control) following pretreatment with PDB plus GnRH, but not PDB alone (Fig. 9); antagonist binding was not significantly changed by pretreatment with PDB or PDB plus GnRH. In other experiments, incubation of cells with 0.3 μM GnRH for 2 h at 4 °C was found to completely block the

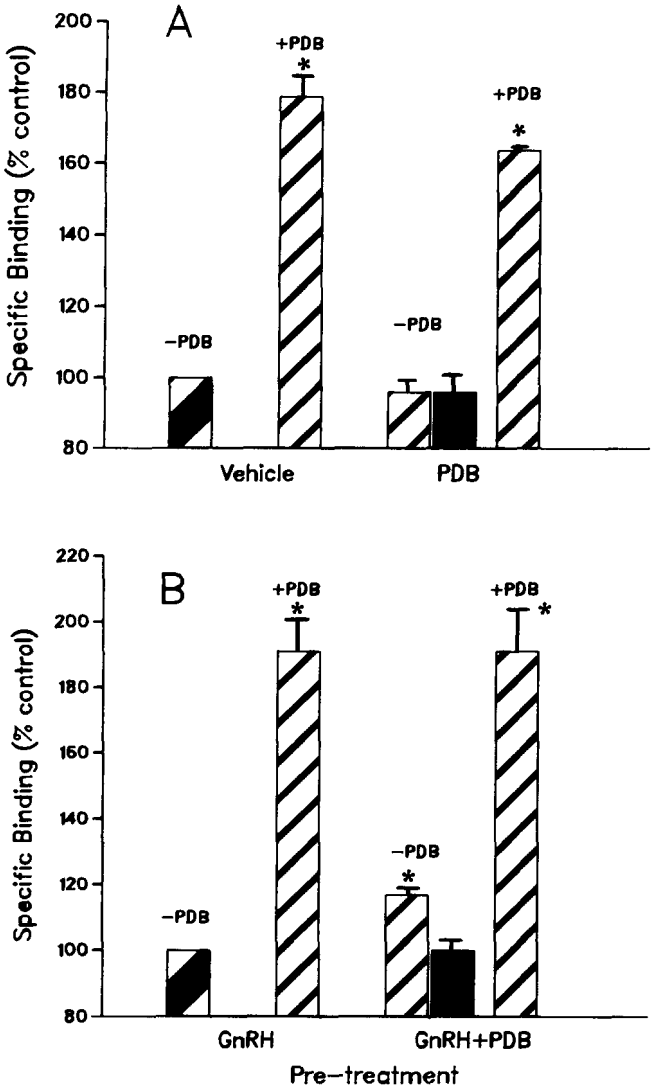

FIG. 8. **Effects of phorbol ester pretreatment on GnRH agonist and antagonist binding.** Pituitary cell cultures were prepared as described under "Experimental Procedures." After 2 days in culture, cells were washed twice with 2 ml/well M199/BSA at 23 °C, then were pretreated for 20 min at 23 °C with either vehicle, 100 nM PDB, 0.3 μM GnRH, or GnRH + PDB (1 ml/well) as indicated. At the end of the pretreatment, cells were repeatedly washed over a 30-min period to remove PDB and/or GnRH as described under "Experimental Procedures." Following this washout period, GnRH receptor binding was measured by incubation (20 min at 23 °C) with a K_d concentration of either the agonist radioligand (*narrow striped bars*) or the antagonist radioligand (*solid bars*), in the presence or absence of 100 nM PDB, as indicated above the bars. Results are expressed as a percent of the specific binding seen in cultures pretreated with vehicle (*panel A*) or GnRH (*panel B*) alone. Binding measured with either the agonist or antagonist radioligand was reduced by 30 ± 3% following GnRH pretreatment alone, reflecting homologous down-regulation of receptors. Results shown are the mean ± S.E. of four separate experiments, each consisting of 2–4 replicate treatment wells. *, significantly different from control, $p < 0.05$.

receptor down-regulation (to 50% of untreated controls) provoked by this concentration of GnRH in cells maintained at 23 °C. The role of receptor internalization in the development of phorbol ester effects on Buserelin binding was further examined by coincubation of radioligand with 100 nM PDB, 150 nM PMA, or 150 nM 4α-PMA for 90 min at 4 °C. Under these conditions, specific binding of Buserelin was increased by a magnitude similar to that seen at 23 °C; 4α-PMA had no effect on binding at 4 °C.

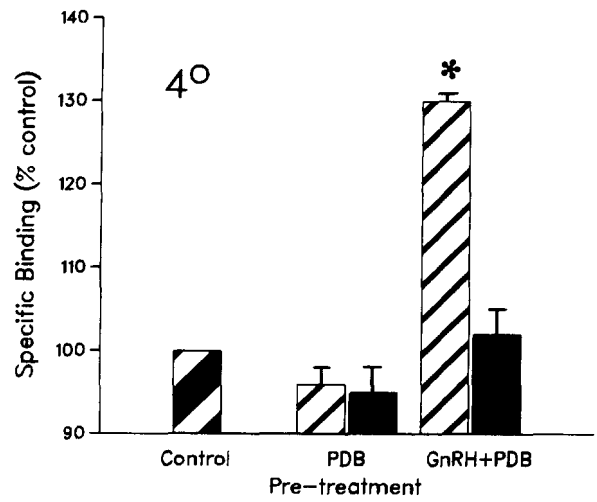

FIG. 9. **Binding of GnRH agonist and antagonist to pretreated cells at 4 °C.** Pituitary cell cultures were pretreated at 23 °C with either vehicle, 100 nM PDB, 0.3 μM GnRH, or GnRH + PDB, and then were washed as described for Fig. 8. The cells were then chilled to 4 °C on ice for 10 min, and GnRH receptor binding was measured by incubation at 4 °C for 90 min (steady-state conditions) with a K_d concentration of either the agonist radioligand (*narrow striped bars*) or the antagonist radioligand (*solid bars*). Results are expressed as a percent of specific binding seen in cultures pretreated with vehicle or GnRH alone. Results shown are the mean ± S.E. of five treatment wells from two separate experiments. Identically pretreated cultures from these same experiments exhibited binding at 23 °C indistinguishable from that shown in Fig. 8. *, significantly different from control, $p < 0.05$.

DISCUSSION

The dose-response relationships for the effects of phorbol esters and dioctanoylglycerol on Buserelin binding observed in the present studies are similar to those associated with other effects of these compounds on gonadotropes, including stimulation of LH release (28, 37, 38) and inhibition of GnRH-stimulated inositol phosphate production (14) in cell cultures, and activation of pituitary protein kinase C *in vitro* (16–18, 28). The 4α-isomer of PMA, which does not activate protein kinase C, stimulate down-regulation of protein kinase C (20), or release LH from cell cultures,[2] also did not enhance Buserelin binding. Similarly, 1,2-dioctanoyl-3-Cl-glycerol, which does not activate protein kinase C or stimulate LH release (28), had no effect on Buserelin binding. It is likely, then, that the effects of phorbol esters and dioctanoylglycerol on Buserelin binding are mediated by protein kinase C, the major intracellular receptor for phorbol esters (39) and exogenous diacylglycerols (40).

The ability to measure GnRH receptors using intact pituitary cells attached to culture plates has permitted the evaluation of GnRH receptor-binding characteristics under conditions similar to those employed for studies of LH release and other cellular responses to GnRH (1, 20, 41). Thus, in the present work, it has been possible to detect an effect of protein kinase C activators on GnRH analog binding which appears to require agonist occupancy of GnRH receptors. This conclusion is supported by several lines of evidence. First, specific binding of a GnRH agonist radioligand, but not that of an antagonist radioligand, was modified when the radioligands were coadministered with a protein kinase C-activating phorbol ester. The agonist and antagonist radioligands were demonstrated to activate and block, respectively, GnRH receptor-dependent LH release and [^{3}H]inositol phosphate production

[2] C. A. McArdle and P. M. Conn, unpublished observations.

under conditions similar to those used to measure receptor binding. Second, Buserelin binding in cell cultures which had been pretreated with PDB, then washed to remove phorbol ester, was not different from binding in vehicle pretreated control cultures, indicating that GnRH receptors are not readily affected if unoccupied during the period of acute protein kinase C activation. Third, previous studies from our laboratory (20) have demonstrated that even prolonged pretreatment (6 h) with a moderate concentration of PMA (20 nM) followed by a 1-h wash does not measurably alter GnRH receptor number or affinity. In this respect, then, GnRH receptors appear to be dissimilar to epidermal growth factor receptors, which are rapidly internalized unoccupied following PMA treatment (22). As a further test of the apparent requirement for agonist occupancy in the effects of protein kinase C activators on GnRH receptors, cells were pretreated with PDB in the presence of GnRH, then washed to remove both PDB and GnRH, and then assayed for GnRH agonist binding with or without readdition of PDB. These experiments revealed that pretreatment with PDB produced residual increases in agonist binding only when GnRH was present during the pretreatment (Fig. 8*B*), which is consistent with a requirement for agonist occupancy of the GnRH receptor while the PKC activator was present. These experiments also demonstrate that the effects of PDB pretreatment (in the presence of GnRH) can persist long enough to be detected by agonist radioligand binding, since it might be argued that the failure to find residual effects of PDB pretreatment alone was due to a rapid reversal of protein kinase C effects.

The modulation of GnRH agonist binding, but not antagonist binding, by protein kinase C activators suggests several possible relationships between the GnRH receptor and protein kinase C. (i) Only the agonist-occupied receptor may assume a conformation necessary for interaction with protein kinase C or its substrate(s). It is possible, for example, that the agonist-occupied form of the GnRH receptor is preferentially phosphorylated by protein kinase C directly, resulting in altered receptor properties. In this regard, it is of interest that *in vitro* phosphorylation of the α_1-adrenergic receptor (another mediator of inositol phospholipid breakdown and Ca^{2+} mobilization in target tissues) by protein kinase C is potentiated by agonist occupancy (42). Further, it is conceivable that the region(s) of the GnRH-binding site so affected by protein kinase C may be accessible (and thus detectable) only by an agonist radioligand. This hypothesis is supported by the finding that binding of Buserelin, but not that of the antagonist, is enhanced following pretreatment of cells with PDB plus GnRH. (ii) The antagonist-occupied receptor may be actively prevented from assuming the requisite conformation for interaction with protein kinase C or its targets. A similar possibility has been suggested by the ability of GnRH receptor antagonists to inhibit GTP-stimulated inositol phospholipid hydrolysis and LH release from permeabilized gonadotropes (43). (iii) Simultaneous activation of agonist-dependent pathways may be required for manifestation of the effects of exogenous protein kinase C activators on agonist binding. Preliminary measurements of antagonist radioligand binding in the presence of varied concentrations of an unlabeled agonist, however, have failed to provide support for this last hypothesis.

The ability of PMA to provoke an increase in Buserelin binding which was resistant to subsequent displacement by excess GnRH is consistent with an action of protein kinase C on GnRH receptor internalization, as shown for the receptors for transferrin (21, 44), epidermal growth factor (22, 45), insulin (46–48), acetylcholine (49), catecholamines (50), and asialoglycoprotein (51). Indeed, an increased rate of agonist-occupied receptor internalization, by decreasing the effective rate of ligand dissociation from intact cells, could account in part for the apparent increase in binding affinity noted in Scatchard analysis. However, other evidence in the present work suggests that this resistance of bound Buserelin to displacement at 4 °C may not be attributable to prior internalization of the receptor-ligand complex. In particular, if phorbol esters increased the extent of agonist-occupied receptor internalization, one would expect that pretreatment with GnRH plus phorbol ester would reduce the binding subsequently measured with a labeled ligand; instead, agonist binding was increased and antagonist binding was unchanged (Fig. 8*B*), suggesting that the rate of receptor internalization had been neither increased nor decreased. In addition, Buserelin binding was enhanced even under conditions (4 °C) which completely block GnRH-stimulated receptor down-regulation, both in cells coincubated with Buserelin and phorbol esters, and in cells pretreated with PDB plus GnRH (Fig. 9). In sum, these results suggest that receptor internalization is not required for either development or subsequent manifestation of phorbol ester effects on GnRH agonist binding and that the association of ligand and receptor, rather than the subsequent processing of receptor/ligand complex, is the step affected by protein kinase C activators. The additional possibility remains, however, that protein kinase C may promote a sequestration (52) of agonist-occupied receptors which is distinct from GnRH-activated receptor internalization.

An increase in apparent B_{max} for Buserelin in the presence of PMA also was observed in the present studies. PMA could affect available numbers of binding sites by stimulating the expression of new cell surface receptors. The rapid (10–20 min) appearance of PMA effects on Buserelin binding suggests that *de novo* synthesis of receptors, such as that reported for epidermal growth factor receptors (53), is an unlikely mechanism. Alternatively, PMA may promote the unmasking of cryptic receptors (54) or increase the basal rate of receptor recycling to the cell surface (10, 11). Neither of these mechanisms, however would easily account for the observed differences between agonist and antagonist binding, and increased recycling of receptors would not be expected to persist at 4 °C. The precise nature of PMA actions on Buserelin binding, then, remains undetermined.

While it is unclear whether the effects of protein kinase C activation on Buserelin binding reflect a pathway normally activated following GnRH binding, the present findings do provide a new experimental approach for the study of GnRH receptor/effector interactions. The role of GTP-binding proteins (G-proteins) in signal transduction and modulation of receptor binding affinity, long recognized in adenylate cyclase-coupled systems (55), has recently gained more widespread appreciation in systems linked to Ca^{2+} mobilization and inositol phospholipid hydrolysis (56). Previous studies from our laboratory have provided evidence that the GnRH receptor is coupled to LH release and inositol phosphate production through a G-protein (43) and that these pathways may be perturbed by receptor blockade or protein kinase C activation. For example, GTP-stimulated LH release and inositol phosphate production in permeabilized gonadotropes can be blocked by a GnRH antagonist (43), and protein kinase C activators can uncouple GnRH receptor activation from inositol phospholipid hydrolysis (14). The ability of phorbol esters to inhibit GTP-stimulated inositol phospholipid hydrolysis (56, 57) and to stimulate phosphorylation and inactivation of adenylate cyclase-coupled G-proteins (59, 60), together with the findings that GTP reduces the affinity of

thyrotropin-releasing hormone (61–62) and muscarinic (63) receptors, suggest that the GnRH receptor-associated G-protein is a possible site of both modulation of GnRH receptor binding and uncoupling of inositol phospholipid hydrolysis by protein kinase C activators. The present studies indicate that the GnRH-gonadotrope model system, in which the interaction of effectors with either agonist- or antagonist-occupied receptors may be measured by a variety of means, may be well-suited to assess the degree of functional analogy between adenylate cyclase- and Ca^{2+}-linked signal transduction systems.

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